

## IgA NEPHROPATHY-RELATED GENES

### CROSS-REFERENCE TO RELATED APPLICATION

This is a continuation-in-part application of PCT/JP97/04468 filed on December 5, 1997.

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

The present invention relates to a novel DNA whose expression level fluctuates in leukocytes of IgA nephropathy patients in comparison with leukocytes of healthy persons, a process for isolating the DNA, a method for detecting the DNA, a novel protein encoded by the DNA, an antibody recognizing the protein, a method for detecting the protein, and diagnosis and treatment of IgA nephropathy.

#### 2. Brief Description of the Background Art

IgA nephropathy is a chronic glomerulonephritis which is characterized in that an IgA immune complex considered to be originated from blood deposits in glomerulus of the kidney. In Japan, the IgA nephropathy occupies 30% or more of primary renal diseases, having the highest frequency as a single renal disease, and 15 to 30% of the disease becomes renal insufficiency due to poor prognosis. However, since the cause of the disease of IgA nephropathy is still unclear, a

fundamental therapeutic method has not been found. Additionally, definite diagnosis of IgA nephropathy imposes heavy burden on patients, because the method is carried out by taking out a portion of the kidney by biopsy and recognizing deposition of the IgA immune complex in mesangium by means of an immunological staining.

It has been reported that about 50% of the patients with IgA nephropathy have a high blood IgA level [*Diseases of the Kidney*, 5th edition (1993), *Nephron*, 29, 170 (1981)]. It is considered that B cells relate to the production of IgA in blood and T cells relate to the regulation of the production. Furthermore, it has been reported that the production of cytokine, such as interleukin 4, interleukin 5, interleukin 6 or TGF- $\beta$  (transforming growth factor- $\beta$ ), is high in peripheral T cells of IgA nephropathy patients in comparison with healthy persons [*Clinical & Experimental Immunology*, 103, 125 (1996), *Kidney International*, 46, 862 (1994)] and that integrin, such as VLA (very late activation)-4 and VLA-5, are strongly activated in peripheral lymphocytes of IgA nephropathy patients [*Nephrology, Dialysis, Transplantation*, 10, 1342 (1995)]. On the basis of these facts, it is considered that, in IgA nephropathy, the production of IgA becomes excess due to abnormality in the immune system, the resulting IgA immune complex in blood deposits on the glomerulus, and activation of the complement system caused

theraby and the like exert influence upon disorders of the glomerulus, but the cause of IgA nephropathy has not been reported.

Elucidation of the cause of IgA nephropathy and its treatment or diagnosis which can reduce a burden on patients are expected.

#### SUMMARY OF THE INVENTION

Accordingly, the present invention provides the development of a novel DNA related to IgA nephropathy, a method for obtaining the DNA, a novel protein related to IgA nephropathy, a method for producing the protein, an antibody recognizing the protein, and a therapeutic drug and a diagnostic drug using the above-described protein, DNA or antibody.

Specifically, the present invention relates to:

- (1) a DNA related to IgA nephropathy, comprising a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:1 to NO:32 and SEQ ID NO:39 to NO:42, or a DNA which hybridizes with said DNA under stringent conditions;
- (2) a DNA comprising a nucleotide sequence identical to continuous 5 to 60 residues in a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:1 to

NO:32 and SEQ ID NO:39 to NO:42, or a DNA comprising a sequence complementary to said DNA;

(3) a DNA comprising a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:43 to NO:104;

(4) a method for detecting mRNA of an IgA nephropathy-related gene using the DNA according to any one of the above (1) to (3);

(5) an IgA nephropathy diagnostic agent comprising the DNA according to any one of the above (1) to (3);

(6) a method for inhibiting transcription of an IgA nephropathy-related gene or translation of mRNA of an IgA nephropathy-related gene using the DNA according to the above (2) or (3);

(7) an IgA nephropathy therapeutic agent comprising the DNA according to the above (2) or (3);

(8) a method for isolating a DNA related to IgA nephropathy from leukocytes of a patient with IgA nephropathy comprising conducting a differential display method;

(9) a protein comprising an amino acid sequence selected from the amino acid sequences represented by SEQ ID NO:33 to NO:38; or a protein comprising an amino acid sequence in which one or several amino acids are deleted, substituted or added in the amino acid sequence of said protein, and having an activity related to IgA nephropathy;

- (10) a DNA encoding the protein according to the above (9);
- (11) a recombinant DNA obtained by inserting the DNA according to the above (10) into a vector;
- (12) a transformant obtained by introducing the recombinant DNA according to the above (11) into a host cell;
- (13) a method for producing the protein according to the above (9), comprising: culturing the transformant according to the above (12) in a medium to produce and accumulate said protein in the culture; and recovering said protein from the resulting culture;
- (14) an antibody which recognizes the protein according to the above (9);
- (15) a method for immunologically detecting the protein according to the above (9) using the antibody according to the above (14);
- (16) an IgA nephropathy diagnostic agent comprising the antibody according to the above (14);
- (17) an IgA nephropathy therapeutic agent comprising the antibody according to the above (14);
- (18) a composition comprising the DNA according to any one of the above (1) to (3) and a diagnostic acceptable carrier;
- (19) a composition comprising the DNA according to the above (2) or (3) and a pharmaceutical acceptable carrier;

(20) a composition comprising the antibody according to the above (14) and a diagnostic acceptable carrier; and

(21) a composition comprising the antibody according to the above (14) and a pharmaceutical acceptable carrier.

The DNA which hybridizes under stringent conditions with a DNA comprising a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:1 to NO:32 and SEQ ID NO:39 to NO:42 means a DNA which is obtained by colony hybridization, plaque hybridization, Southern blot hybridization or the like using, as a probe, a DNA comprising a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:1 to NO:32 and SEQ ID NO:39 to NO:42. Examples include DNA which can be identified by carrying out

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hybridization at 65°C in the presence of 0.7-1.0 M NaCl using a filter on which a DNA prepared from colonies or plaques is immobilized, and then washing the filter with 0.1 x to 2 x SSC solution (the composition of 1 x SSC comprises 150 mM sodium chloride and 15 mM sodium citrate) at 65°C.

The hybridization can be carried out in accordance with known methods described in, for example, *Molecular Cloning, A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press (1989) (referred to as "Molecular Cloning, 2nd ed." hereinafter), *Current Protocols in Molecular Biology*, John Wiley & Sons (1987-1997) (referred to as "Current Protocols in Molecular Biology" hereinafter), *DNA Cloning 1: Core Techniques, A Practical Approach*, Second Edition, Oxford University (1995) or the like. Specific examples of the DNA which can be hybridized include a DNA having a homology of 60% or more with a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:1 to NO:32 and SEQ ID NO:39 to NO:42, preferably a DNA having a homology of 80% or more, and more preferably a DNA having a homology of 95% or more.

Also, the DNA of the present invention includes an oligonucleotide and antisense oligonucleotide containing a partial sequence of the IgA nephropathy-related DNA.

Examples of the oligonucleotide include oligonucleotides comprising a sequence identical to a

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sequence of continuous 5 to 60 residues, preferably continuous 10 to 50 residues, in a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:1 to NO:32 and SEQ ID NO:39 to NO:42. Examples of the antisense oligonucleotide include antisense oligonucleotides of the oligonucleotides. Specific examples include oligonucleotides comprising a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:43 to NO:104.

Examples of the protein of the present invention include proteins having an activity related to IgA nephropathy. Specific examples include a protein comprising an amino acid sequence selected from the amino acid sequences represented by SEQ ID NO:33 to NO:38, and a protein comprising an amino acid sequence in which one or several amino acids are deleted, substituted or added in the amino acid sequence of said protein and having an activity related to IgA nephropathy.

The protein comprising an amino acid sequence in which one or several amino acids are deleted, substituted or added in the amino acid sequence of the protein that has an amino acid sequence selected from the amino acid sequences represented by SEQ ID NO:33 to NO:38 and having an activity related to IgA nephropathy can be prepared in accordance with known methods described in, for example, *Molecular Cloning*,



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2nd ed., *Current Protocols in Molecular Biology, Nucleic Acids Research*, 10, 6487 (1982), *Proc. Natl. Acad. Sci. USA*, 79, 6409 (1982), *Gene*, 34, 315 (1985), *Nucleic Acids Research*, 13, 4431 (1985), *Proc. Natl. Acad. Sci. USA*, 82, 488 (1985) and the like.

Examples of the antibody of the present invention include antibodies which recognize the above-described proteins.

The present invention is described in detail.

#### 1. Preparation of IgA nephropathy-related DNA

Taking note of the difference in the expression quantity of mRNA in leukocytes between patients with IgA nephropathy and healthy persons, the IgA nephropathy-related DNA is isolated using the differential display method [*FEBS Letters*, 351, 231 (1994)]. That is, an amplified cDNA fragment of a novel gene (referred to as "IgA nephropathy-related gene" hereinafter) whose expression level increases or decreases significantly in leukocytes of a patient with IgA nephropathy as compared with leukocytes of a healthy person is obtained by subjecting total RNA or mRNA extracted from cells to the polymerase chain reaction (PCR) using various primers.

This method is described below.

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Total RNA or mRNA is prepared from leukocytes of patients with IgA nephropathy and leukocytes of healthy persons.

Examples of the method for the preparation of total RNA include guanidine thiocyanate-caesium trifluoroacetate method [Methods in Enzymol., 154, 3 (1987)] and the like.

Examples of the method for preparing poly(A)<sup>+</sup> RNA from total RNA include oligo(dT)-immobilized cellulose column method (Molecular Cloning, 2nd ed.) and the like.

The mRNA can be also prepared using a kit, such as Fast Track mRNA Isolation Kit (manufactured by Invitrogen), Quick Prep mRNA Purification Kit (manufactured by Pharmacia) or the like.

Using an anchor primer, cDNA is synthesized in the usual way from the RNA extracted by the above-described method from leukocytes of a patient with IgA nephropathy or leukocytes of a healthy person, and then the cDNA is amplified by subjecting it to PCR using an anchor primer having a 5'-end labeled with fluorescence and an arbitrary primer.

The anchor primer is a primer in which an oligonucleotide of adenine, guanine or cytosine, excluding thymidine, is added to the 3'-end of an oligo(dT) sequence which hybridizes with a 3'-end poly(A) sequence of mRNA, and



(1977)] or using a nucleotide sequence analyzer such as 373A DNA Sequencer (manufactured by Perkin Elmer).

Examples of the vector used for the integration of the amplified DNA fragment include pBluescript KS(+) (manufactured by Stratagene), pDIRECT [Nucleic Acids Research, 18, 6069 (1990)], pPCR-Script Amp [manufactured by Stratagene, Strategies, 5, 6264 (1992)], pT7Blue (manufactured by Novagen), pCR II [manufactured by Invitrogen, Biotechnology, 2, 657 (1991)], pCR-TRAP (manufactured by GenesHunter), pNotA<sub>TT</sub> (manufactured by 5'→3') and the like.

Novelty of the nucleotide sequence determined in this manner can be verified by searching a data base, such as GenBank, EMBL, DDBJ and the like, using a homology searching program, such as blast and the like, thereby finding that there is no nucleotide sequence which shows an obvious homology that coincides with the nucleotide sequences in the data base.

Examples of the thus obtained partial DNA fragment of cDNA of the IgA nephropathy-related gene include DNA comprising a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:7 to NO:32 and SEQ ID NO:39 to NO:42.

When the DNA obtained by the above-described method is a partial DNA fragment of cDNA which corresponds to IgA nephropathy-related mRNA, full-length cDNA can be obtained by

the following method (1) or (2) using the DNA obtained by the above-described method.

(1) Application of cDNA library

A full-length cDNA can be obtained by carrying out screening according to hybridization using the above-described DNA fragment as the probe and various cDNA libraries.

The method for the preparation of cDNA libraries is described below.

Examples of the method for the preparation of cDNA libraries include methods described in *Molecular Cloning*, 2nd. ed., *Current Protocols in Molecular Biology*, or *DNA Cloning 1: Core Techniques, A Practical Approach*, Second Addition, Oxford University Press (1995), or methods using a commercially available kit, such as SUPERScript Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by Life Technologies) or ZAP-cDNA Synthesis Kit (manufactured by Stratagene). Additionally, commercially available cDNA libraries, such as a human leukocyte cDNA library (manufactured by Life Technologies) and the like, can be also used.

In preparing the cDNA library, any one of phage vectors, plasmid vectors and the like can be used as the cloning vector which replicates autonomously in *Escherichia coli* K12. Examples include ZAP Express (manufactured by

Stratagene, *Strategies*, 5, 58 (1992)), pBluescript II SK(+) [Nucleic Acids Research, 17, 9494 (1989)],  $\lambda$  ZAP II (manufactured by Stratagene),  $\lambda$ gt10,  $\lambda$ gt11 [DNA Cloning, A Practical Approach, 1, 49 (1985)],  $\lambda$ ExCell (manufactured by Pharmacia), pCD2 [Mol. Cell. Biol., 3, 280 (1983)], pUC18 [Gene, 33, 103 (1985)], and the like.

With regard to the *Escherichia coli* used to transform with the vector containing the cDNA, any microorganism belonging to *Escherichia coli* can be used. Examples include *Escherichia coli* XL1-Blue MRF' [manufactured by Stratagene, *Strategies*, 5, 81 (1992)], *Escherichia coli* C600 [Genetics, 39, 440 (1954)], *Escherichia coli* Y1088 [Science, 222, 778 (1983)], *Escherichia coli* Y1090 [Science, 222, 778 (1983)], *Escherichia coli* NM522 [J. Mol. Biol., 166, 1 (1983)], *Escherichia coli* K802 [J. Mol. Biol., 16, 118 (1966)], *Escherichia coli* JM105 [Gene, 38, 275 (1985)], and the like.

A cDNA clone can be selected from the cDNA library according to a colony hybridization or plaque hybridization method (*Molecular Cloning*, 2nd ed.) using a probe labeled with an isotope or digoxigenin.

The DNA of interest can be obtained from the thus selected clone in the usual way.

(2) The DNA of interest can be also obtained by the 5'-RACE (rapid amplification of cDNA ends) and 3'-RACE method [Proc. Natl. Acad. Sci. USA, 85, 8998 (1988)] in which cDNA

is synthesized from mRNA by the above-described method, adapters are added to both ends of the cDNA and then PCR is carried out using primers based on the nucleotide sequence of the adapter and the nucleotide sequence of the amplified fragment.

Nucleotide sequence of the DNA obtained by these methods can be determined by the above-described nucleotide sequence determining method. Novelty of the sequence can be also verified by the above-described method.

Examples of the full-length cDNA of the IgA nephropathy-related gene obtained in this manner include DNAs having the nucleotide sequences represented by SEQ ID NO:1 to NO:6.

Once a DNA of IgA nephropathy-related gene is obtained and a nucleotide sequence thereof is determined in the above-described manner, the DNA of interest can be obtained by PCR [PCR Protocols, Academic Press (1990)] by preparing primers based on the nucleotide sequence and using cDNA synthesized from the mRNA or a cDNA library as the template. Alternatively, the DNA of interest may be prepared by chemical synthesis using a DNA synthesizer based on the determined DNA nucleotide sequence. Examples of the DNA synthesizer include DNA Synthesizer Model 392 (manufactured by Perkin-Elmer) using the phosphoramidite method.

On the basis of the nucleotide sequence information of the above-described DNA and DNA fragments, an oligonucleotide having a partial sequence of the IgA nephropathy-related DNA and a corresponding antisense oligonucleotide can be prepared.

Examples of the oligonucleotide or antisense oligonucleotide include a sense primer corresponding to a 5'-end side nucleotide sequence, and an antisense primer corresponding to a 3'-end side nucleotide sequence, of a portion of the mRNA to be detected. In this case, the base corresponding to uracil in mRNA corresponds to thymidine in the oligonucleotide primer.

As the sense primer and antisense primer, it is preferred to use oligonucleotides in which melting point ( $T_m$ ) and the number of bases are not significantly different from each other, and those which have 5 to 60 bases, preferably 10 to 50 bases, can be used.

Also, an analogue of the oligonucleotide can be used in the present invention. For example, the methyl or phosphorothioate analogue of the oligonucleotide may be used.

Examples of the oligonucleotide or antisense oligonucleotide comprising a partial sequence of the IgA nephropathy-related DNA include an oligonucleotide comprising a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:43 to NO:104.



## 2. Production of protein having an activity related to IgA nephropathy

The full-length cDNA of IgA nephropathy-related gene obtained by the method described in the above section 1 encodes a protein having an activity related to IgA nephropathy (referred to as "IgA nephropathy-related protein" hereinafter). The IgA nephropathy-related protein is prepared by expressing the IgA nephropathy-related gene in a host cell as shown below. A DNA fragment having a suitable length containing a portion encoding the protein is prepared from the full-length cDNA as occasion demands. An expression plasmid of the protein is prepared by inserting the DNA fragment or the full-length cDNA into a downstream site of the promoter in the expression vector. The expression plasmid is introduced into a host cell suitable for the expression vector.

As the host cell, any cell can be used so long as it can express the gene of interest. Examples include bacteria belonging to the genus *Escherichia*, *Serratia*, *Corynebacterium*, *Brevibacterium*, *Pseudomonas*, *Bacillus*, *Microbacterium* and the like, yeasts belonging to the genus *Kluyveromyces*, *Saccharomyces*, *Schizosaccharomyces*, *Trichosporon*, *Schwanniomyces* and the like, animal cells, insect cells, and the like.

Examples of the expression vector include those which can replicate autonomously in the just described host cell or can be integrated into chromosome and have a promoter at such a position that the IgA nephropathy-related gene can be transcribed.

When a bacterium or the like is used as the host cell, it is preferred that the IgA nephropathy-related gene expression vector can replicate autonomously in the bacterium and is a recombinant vector constructed with a promoter, a ribosome binding sequence, the IgA nephropathy-related gene and a transcription termination sequence. A promoter controlling gene may also be contained.

Examples of the expression vector include pBTrp2, pBTac1 and pBTac2 (all available from Boehringer Mannheim Co.), pKK233-2 (manufactured by Pharmacia), pSE280 (manufactured by Invitrogen), pGEMEX-1 (manufactured by Promega), pQE-8 (manufactured by QIAGEN), pKYP10 (Japanese Published Unexamined Patent Application No. 110600/83), pKYP200 [*Agric. Biol. Chem.*, 48, 669 (1984)], pLSA1 [*Agric. Biol. Chem.*, 53, 277 (1989)], pGEL1 [*Proc. Natl. Acad. Sci. USA*, 82, 4306 (1985)], pBluescript II SK(-) (manufactured by Stratagene), pGEX (manufactured by Pharmacia), pET-3 (manufactured by Novagen), pTerm2 (U.S. Patents 4,686,191, 4,939,094 and 5,160,735), pUB110, pTP5, pC194, pEG400 [*J. Bacteriol.*, 172, 2392 (1990)] and the like.

With regard to the promoter, any promoter can be used so long as it can drive the expression in the host cell. Examples include promoters originated from *Escherichia coli*, phage and the like (for example, trp promoter ( $P_{trp}$ ), lac promoter ( $P_{lac}$ ),  $P_L$  promoter,  $P_R$  promoter, T7 promoter and the like), SPO1 promoter, SPO2 promoter, penP promoter and the like. Also, artificially designed and modified promoters, such as a promoter in which two  $P_{trp}$  are linked in series ( $P_{trp} \times 2$ ), tac promoter, letI promoter [Gene, 44, 29 (1986)] and lacT7 promoter and the like, can be used.

With regard to the ribosome binding sequence, any sequence can be used so long as it can effect the expression in the host cell. However, it is preferred to use a plasmid in which the space between Shine-Dalgarno sequence and the initiation codon is adjusted to an appropriate distance (for example, 6 to 18 bases).

Production efficiency of the protein of interest can be improved by substituting a base in a nucleotide sequence which encodes the IgA nephropathy protein of the present invention so as to form a codon suitable for the expression of a host.

The transcription termination sequence is not always necessary for the expression of the IgA nephropathy-related gene of the present invention. However, it is preferred to

arrange the transcription terminating sequence at just downstream of the structural gene.

Examples of the host cell include microorganisms belonging to the genus *Escherichia*, *Serratia*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Microbacterium*, *Pseudomonas*, and the like. Specific examples include *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* MC1000, *Escherichia coli* KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No.49, *Escherichia coli* W3110, *Escherichia coli* NY49, *Serratia ficaria*, *Serratia fonticola*, *Serratia liquefaciens*, *Serratia marcescens*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Brevibacterium ammoniagenes*, *Brevibacterium immariophilum* ATCC 14068, *Brevibacterium saccharolyticum* ATCC 14066, *Corynebacterium glutamicum* ATCC 13032, *Corynebacterium glutamicum* ATCC 14067, *Corynebacterium glutamicum* ATCC 13869, *Corynebacterium acetoacidophilum* ATCC 13870, *Microbacterium ammoniophilum* ATCC 15354, *Pseudomonas* sp. D-0110 and the like.

With regard to the method for the introduction of the recombinant vector, any one of the known methods for introducing DNA into the just described host cells, such as a method in which calcium ion is used [Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)], a protoplast method (Japanese Published Unexamined Patent Application No. 2483942/88), the



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Unexamined Patent Application No. 227075/90), pcDM8 [Nature, 329, 840 (1987)], pcDNA1/Amp (manufactured by Invitrogen), pREP4 (manufactured by Invitrogen), pAGE103 [J. Biochem., 101, 1307 (1987)], pAGE210 and the like can be exemplified as the expression vector.

Any promoter can be used so long as it can drive the expression in animal cell. Examples include a promoter of IE (immediate early) gene of cytomegalovirus (CMV), an early promoter of SV40, a promoter of retrovirus, a metallothionein promoter, a heat shock promoter, an SRO promoter and the like. Also, the enhancer of the IE gene of human CMV may be used together with the promoter.

Examples of the host cell include human Namalwa cell, monkey COS cell, Chinese hamster CHO cell, HST5637 (Japanese Published Unexamined Patent Application No. 299/88), and the like.

With regard to the method for the introduction of the recombinant vector into animal cells, any one of the known methods for introducing DNA into animal cells, such as an electroporation method [Cytotechnology, 3, 133 (1990)], a calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), a lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)] and the method described in Virology, 52, 456 (1973), can be used. Preparation and culturing of transformants can be carried out

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in accordance with the method described in Japanese Published Unexamined Patent Application No. 227075/90 or Japanese Published Unexamined Patent Application No. 257891/90.

When an insect cell is used as the host cell, the protein can be expressed by known methods described in, for example, *Baculovirus Expression Vectors, A Laboratory Manual, Current Protocols in Molecular Biology*, supplement 1-38 (1987-1997) *Bio/Technology*, 6, 47 (1988), or the like.

That is, a recombinant gene transfer vector and baculovirus are simultaneously inserted into an insect cell to obtain a recombinant virus in an insect cell culture supernatant, and then the insect cells are infected with the thus obtained recombinant virus to effect expression of the protein.

Examples of the gene introducing vector used in the method include pVL1392, pVL1393, pBlueBacIII (all manufactured by Invitrogen), and the like.

Examples of the baculovirus include *Autographa californica* nuclear polyhedrosis virus with which insects of the family *Barathra* are infected, and the like.

Examples of the insect cell include *Spodoptera frugiperda* oocytes Sf9 and Sf21 (*Baculovirus Expression Vectors, A Laboratory Manual*, W.H. Freeman and Company, New York, (1992)), *Trichoplusia ni* oocyte High 5 (manufactured by Invitrogen) and the like.

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The method for the co-transfer of the above-described recombinant gene transfer vector and the above-described baculovirus for the preparation of the recombinant virus include calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)] and the like.

With regard to the gene expression method, a secretion production, a fusion protein expression and the like can be effected in accordance with the method described in *Molecular Cloning*, 2nd ed., in addition to the direct expression.

When expressed in a yeast, an animal cell or a insect cell, a glycosylated protein can be obtained.

The IgA nephropathy-related protein can be produced by culturing a transformant comprising a recombinant DNA containing the IgA nephropathy-related gene in a culture medium to produce and accumulate the IgA nephropathy-related protein, and recovering the protein from the resulting culture.

Culturing of the transformant used in the production of the IgA nephropathy-related protein of the present invention in a culture medium is carried out in accordance with a usual method used in culturing of respective host cells.



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When the transformant of the present invention is an prokaryote, such as *Escherichia coli* or the like, or an eukaryote, such as yeast or the like, the medium used in culturing of these microorganisms may be either a natural medium or a synthetic medium, so long as it contains a carbon source, a nitrogen source, an inorganic salt and the like which can be assimilated by the microorganisms and can perform culturing of the transformant efficiently.

Examples of the carbon source include those which can be assimilated by respective microorganisms, such as carbohydrates (for example, glucose, fructose, sucrose, molasses containing them, starch, starch hydrolysate, and the like), organic acids (for example, acetic acid, propionic acid, and the like), and alcohols (for example, ethanol, propanol, and the like).

Examples of the nitrogen source include ammonia, various ammonium salts of inorganic acids or organic acids (for example, ammonium chloride, ammonium sulfate, ammonium acetate, ammonium phosphate, and the like), other nitrogen-containing compounds, peptone, meat extract, yeast extract, corn steep liquor, casein hydrolysate, soybean meal and soybean meal hydrolysate, various fermented cells and hydrolysates thereof, and the like.

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Examples of inorganic substance used in the culture medium include potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate, and the like.

The culturing is carried out under aerobic conditions by shaking culture, aeration stirring culture or the like means. The culturing temperature is preferably from 15 to 45°C, and the culturing time is generally from 16 hours to seven days. The pH of the medium is maintained at 3.0 to 9.0 during the culturing. Adjustment of the medium pH is carried out using an inorganic or organic acid, an alkali solution, urea, calcium carbonate, ammonia and the like.

Also, antibiotics (for example, ampicillin, tetracycline, and the like) may be added to the medium during the culturing as occasion demands.

When a microorganism transformed with an expression vector containing an inducible promoter is cultured, an inducer may be added to the medium as occasion demands. For example, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) or the like may be added to the medium when a microorganism transformed with an expression vector containing lac promoter is cultured, or indoleacrylic acid (IAA) or the like may be added thereto when a microorganism transformed with an expression vector containing trp promoter is cultured.



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Additionally, antibiotics (for example, gentamicin, and the like) may be added to the medium during the culturing as occasion demands.

When the protein of the present invention having an activity related to IgA nephropathy is isolated and purified from a culture of the transformant of the present invention, usual methods for the isolation and purification of enzymes may be used.

For example, when the protein of the present invention is expressed in a dissolved state inside the cells, the cells after completion of the culturing are recovered by centrifugation, suspended in a buffer of aqueous system and then disrupted using ultrasonic oscillator, French press, Manton Gaulin homogenizer, dyncmill or the like to obtain a cell-free extract. A purified product can be obtained from a supernatant fluid prepared by centrifugation of the cell-free extract, by employing a technique or a combination of techniques, such as solvent extraction, salting out with ammonium sulfate or the like, desalting, precipitation with organic solvents, anion exchange chromatography using a resin (for example, diethylaminoethyl (DEAE)-Sephadex, DIAION HPA-75 (manufactured by Mitsubishi Chemical), or the like), cation exchange chromatography using a resin (for example, S-Sephadex FF (manufactured by Pharmacia), or the like), hydrophobic chromatography using a resin (for example, butyl-



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Examples of the protein obtained in this manner include proteins having an amino acid sequence selected from the amino acid sequences represented by SEQ ID NO:33 to NO:38.

Additionally, the protein expressed by the above-described method can be produced by a chemical synthesis method, such as Fmoc method (fluorenylmethyloxycarbonyl method), tBoc method (t-butyloxycarbonyl method) or the like. It can be also synthesized using a peptide synthesizer available from Sowa Boeki (manufactured by Advanced chemTech, USA), Perkin-Elmer Japan (manufactured by Perkin-Elmer, USA), Pharmacia Biotech (manufactured by Pharmacia Biotech, Sweden), Aroka (manufactured by Protein Technology Instrument, USA), KURABO (manufactured by Synthecell-Vega, USA), Japan PerSeptive Limited (manufactured by PerSeptive, USA) or Shimadzu Corporation.

### 3. Preparation of antibody which recognizes the protein of the present invention

A purified product of the whole length or a partial fragment of the protein obtained by the method described in the above section 2 or a peptide having a partial amino acid sequence of the protein of the present invention is used as the antigen. The antigen is administered to animal by intravenous or intraperitoneal injection together with an

appropriate adjuvant (for example, complete Freund's adjuvant, aluminum hydroxide gel, pertussis vaccine, or the like).

Examples of the animals used include rabbits, goats, 3- to 20-week-old rats, mice, hamsters and the like.

Preferable dosage of antigen is 50 to 100 µg per animal.

When a peptide is used as the antigen, it is preferred to use the peptide as the antigen after binding it covalently to a carrier protein, such as keyhole limpet haemocyanin, bovine thyroglobulin or the like. The peptide used as the antigen can be synthesized using a peptide synthesizer.

Administration of the antigen is carried out 3 to 10 times at one- to two-week intervals after the first administration. A blood sample is recovered from the fundus of the eye 3 to 7 days after each administration, and the serum is tested, for example, by enzyme immunoassay (Enzyme-linked Immunosorbent Assay (ELISA), published by Igaku Shoin (1976); *Antibodies - A Laboratory Manual*, Cold Spring Harbor Laboratory (1988)) as to whether it is reactive with the antigen used for immunization. A non-human mammal whose serum shows a sufficient antibody titer against the antigen used for immunization is submitted for use as the supply source of serum or antibody producing cells.

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A polyclonal antibody can be prepared by isolating and purifying it from the serum.

A monoclonal antibody can be prepared by preparing a hybridoma through fusion of the antibody producing cells with myeloma cells of a non-human mammal and culturing the hybridoma, or administering the hybridoma to an animal to induce ascites tumor in the animal, and then isolating and purifying it from the culture medium or ascitic fluid.

Examples of the antibody producing cells include spleen cells, lymph nodes and antibody producing cells in peripheral blood. Particularly, spleen cells are preferred.

Examples of the myeloma cells include cell lines derived from mouse, such as P3-X63Ag8-U1 (P3-U1) cell line [Current Topics in Microbiology and Immunology, 18, 1-7 (1978)], P3-NS1/1-Ag41 (NS-1) cell line [European J. Immunology, 6, 511-519 (1976)], SP2/O-Ag14 (SP-2) cell line [Nature, 276, 269-270 (1978)], P3-X63-Ag8653 (653) cell line [J. Immunology, 123, 1548-1550 (1979)], P3-X63-Ag8 (X63) cell line [Nature, 256, 495-497 (1975)] and the like, which are 8-azaguanine-resistant mouse (BALB/c) myeloma cell lines.

Hybridoma cells can be prepared in the following manner.

Antibody producing cells and myeloma cells are fused, suspended in HAT medium (normal medium supplemented with hypoxanthine, thymidine and aminopterin) and then cultured





For example, diagnosis of IgA nephropathy may be effected by carrying out PCR using the DNA described in the above section 1 which corresponds to the mRNA to be detected as a pair of oligonucleotide primers and detecting the amplified fragment. In that case, the nucleotide sequence moiety to be amplified may be any nucleotide sequence region of the mRNA, but a nucleotide sequence region which has a length of from 50 bp to 2 kbp and does not contain a sequence rich in a repeating sequence or GC (guanine-cytosine) bases is preferred.

(2) Using the antisense oligonucleotide (RNA/DNA) described in the above section 1 [Chemistry, 46, 681 (1991), Biotechnology, 9, 358 (1992)], treatment of IgA nephropathy can be effected by inhibiting transcription of DNA or translation of mRNA.

An example of the antisense oligonucleotide (RNA/DNA) of the above section 1 used in this case is an antisense oligonucleotide which has a partial nucleotide sequence, preferably a sequence of from 10 to 50 bases in the translation initiation region, of a DNA which encodes the protein described in the above section 2.

(3) Using the DNA described in the above section 1, the IgA nephropathy-related protein of the present invention can be obtained by the method described in the above section 2.

(4) Using the protein described in the above section 2 as the antigen, antibodies can be produced by the method described in the above section 3.

(5) Using the antibody described in the above section 3, the IgA nephropathy-related protein can be detected or determined immunologically.

Examples of the immunological detection method include ELISA method using a microtiter plate, fluorescent antibody technique, western blot technique, immunohistochemical staining and the like.

Examples of the immunological determination method include sandwich ELISA method in which, among antibodies which react with the protein of the present invention in solution, two monoclonal antibodies having different epitopes are used and radioimmunoassay method in which the protein of the present invention labeled with radioactive isotope, such as  $^{125}\text{I}$  or the like, and an antibody which recognizes the protein of the present invention are used.

(6) Using the antibody described in the above section 3, the presence or absence of IgA nephropathy in a person to be inspected can be diagnosed by immunologically detecting or determining an IgA nephropathy-related protein in leukocytes collected from a healthy person and the person to be inspected, comparing its amounts in the healthy person and person to be inspected and then examining the quantitative

fluctuati n. As a specific sample to be tested, leukocytes separated from peripheral blood samples of a healthy person and a person to be inspected can be used. Additionally, when the IgA nephropathy-related protein to be detected is a protein secreted from leukocytes, the presence or absence of IgA nephropathy in a person to be inspected can be detected and diagnosed by immunologically detecting or determining the protein in blood plasma samples collected from a healthy person and the person to be inspected, comparing its amounts in the healthy person and person to be inspected and then examining its quantitative fluctuation.

(7) The antibody described in the above section 3 can be applied to the treatment or prevention of IgA nephropathy.

When the DNA, protein and antibody is used for the diagnosis, treatment or prevention of IgA nephropathy, a diagnostically or pharmacologically acceptable carrier may be added.

#### EXAMPLES

Examples of the present invention are given below by way of illustration and not by way of limitation.

Example 1: Differential display of leukocytes of IgA nephropathy patients and healthy persons

(1) Preparation of total RNA from leukocytes of IgA nephropathy patients and healthy persons

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A 20 ml portion of blood was collected from each of five IgA nephropathy patients and five healthy persons.

This was mixed with 500  $\mu$ l of 1,000 units/ml heparin solution to inhibit coagulation, transferred into a centrifugation tube and then centrifuged at 3,300 rpm for 15 minutes at room temperature, and the resulting intermediate layer buffy coat containing leukocytes was transferred into another centrifugation tube.

Thereafter, total RNAs were obtained in accordance with the AGPC method [Experimental Medicine, 9, 1937 (1991)] or using an RNA recovering kit RNeasy (manufactured by QIAGEN).

(2) Fluorescence differential display using leukocyte total RNAs of IgA nephropathy patients and healthy persons

Distilled water was added to 2.5  $\mu$ g of each of the total RNAs obtained in the above step (1) to a total volume of 9  $\mu$ l, and the solution was mixed with 1  $\mu$ l of an anchor primer (50  $\mu$ M, custom-synthesized by Sawady Technology) whose 5'-end had been fluorescence-labeled with fluorescein isothiocyanate (referred to as "FITC" hereinafter), heated at 70°C for 5 minutes and then immediately cooled on an ice bath.

Since each of the three primers FAH (nucleotide sequence is shown in SEQ ID NO:105), FGH (nucleotide sequence is shown in SEQ ID NO:106) and FCH (nucleotide sequence is shown in SEQ ID NO:107) was used in each reaction as the

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5'-end fluorescence-labeled anchor primer, a total of three combinations of reactions were carried out for one sample of total RNAs.

A 4  $\mu$ l portion of 5  $\times$  reverse transcriptase reaction buffer [250 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl<sub>2</sub>] was mixed with 2  $\mu$ l of 100 mM dithiothreitol (DTT), 1  $\mu$ l of 10 mM dNTP (dATP, dGTP, dTTP and dCTP), 1  $\mu$ l of distilled water and 1  $\mu$ l (200 units) of a reverse transcriptase SUPERScript II RNase H<sup>-</sup> Reverse Transcriptase (manufactured by Life Technologies), and the resulting mixture was allowed to stand at room temperature for 10 minutes, allowed to react at 42°C for 50 minutes to synthesize a cDNA, and then heated at 90°C for 5 minutes to terminate the reaction.

After the reaction, to the reaction solution was added 40  $\mu$ l of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM disodium ethylenediaminetetraacetate (EDTA) (pH 8.0)].

Next, 14.7  $\mu$ l of distilled water, 2  $\mu$ l of 10  $\times$  PCR buffer [100 mM Tris-HCl (pH 8.8), 500 mM KCl, 15 mM MgCl<sub>2</sub>, 1% Triton X-100], 0.8  $\mu$ l of 2.5 mM dNTP, 0.3  $\mu$ l of 50  $\mu$ M fluorescence-labeled anchor primer (the same among FAE, FGH and FCH used in the cDNA synthesis), 1  $\mu$ l of 10  $\mu$ M arbitrary primer (manufactured by Operon Technologies) and 0.2  $\mu$ l of DNA polymerase Gene Taq (5 units/ $\mu$ l, manufactured by Nippon Gene) were added to 1  $\mu$ l of each of the thus synthesized cDNA







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Each of the resulting reaction solutions was extracted with phenol-chloroform (1:1) and then with chloroform-isoamyl alcohol (24:1), subsequently carrying out ethanol precipitation.

The thus obtained precipitate (amplified DNA fragments) was dissolved in TE buffer and subjected to 1.5% low melting point agarose gel (SEA PLAQUE GTG, manufactured by FMC Bioproducts) electrophoresis.

After the electrophoresis, the resulting gels were stained with ethidium bromide and then the bands containing amplified fragments were cut off.

The gel was heated at 65°C for 15 minutes to melt agarose and then extracted with phenol-chloroform and then with chloroform-isoamyl alcohol.

The thus obtained extract was subjected to ethanol precipitation and the resulting precipitate (amplified fragments) was dissolved in 10 µl of TE buffer.

A 1 µl portion of each of the amplified fragments was mixed with 1 µl of a vector for PCR fragment cloning use, pT7BlueT-Vector (manufactured by Novagen), and the amplified fragment was cloned into the plasmid using DNA Ligation Kit ver.1 (manufactured by Takara Shuzo) in accordance with the manual attached to the kit.

Using the thus obtained recombinant plasmid, *Escherichia coli* DH5α (manufactured by Gibco BRL) was

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transformed in accordance with a known method, and the resulting transformant was spread on LB agar medium containing 50 µg/ml of ampicillin and cultured overnight at 37°C.

The thus grown ampicillin-resistant transformant was suspended in 20 µl of distilled water, the suspension was mixed with 2.5 µl of 10 × PCR buffer, 2 µl of 2.5 mM dNTP, 0.3 µl of 34 µM anchor primer, 1 µl of 10 µM arbitrary primer and 0.5 µl of a DNA polymerase Gene Taq, and the mixture was subjected to PCR under the same conditions of the above-described re-amplification of amplified fragments and then analyzed by electrophoresis which recognized that an amplified fragment has the same length as in the first differential display.

Nucleotide sequence of the amplified fragment was determined using DNA Sequencer (manufactured by Perkin Elmer). In carrying out the nucleotide sequence determination, Dye Primer Cycle Sequencing Kit manufactured by Perkin Elmer and the method described in the manual attached to the kit were used.

Using restriction enzymes capable of cleaving restriction enzyme sites in the determined nucleotide sequence, the reaction product obtained by the above-described differential display was cleaved and then subjected to electrophoresis to recognize that the position of

electrophoresis band corresponding to the thus cut off amplified fragment was changed.

Each of the thus obtained nucleotide sequences was compared with a nucleotide sequence data base GenBank to select a total of 66 clones which were not present among the known nucleotide sequences in the data base or coincided only with the expressed sequence tag among nucleotide sequences in the data base.

#### Example 2 Detection of specificity of mRNA expression by RT-PCR

Using 2 µg of each of the total RNAs obtained in Example 1 from leukocytes of five cases of IgA nephropathy patients and 5 cases of healthy persons, a single-stranded cDNA was synthesized using a single-stranded cDNA synthesis kit, Superscript Preamplification System (manufactured by Life Technologies) in accordance with the method described in the manual attached to the kit.

A 21 µl portion of the thus obtained solution containing the single-stranded cDNA was adjusted to a total volume of 420 µl by adding distilled water.

Using 10 µl portion of the thus prepared solution, the expression level of mRNA corresponding to each amplified fragment was detected by carrying out RT-PCR in the following manner.

That is, 10  $\mu$ l of the leukocyte single-stranded cDNA solution was mixed with 15.8  $\mu$ l of distilled water, 4  $\mu$ l of 10  $\times$  PCR buffer, 3.2  $\mu$ l of 2.5 mM dNTP, 2  $\mu$ l of DMSO, 2  $\mu$ l of 10  $\mu$ M gene-specific sense primer, 2  $\mu$ l of 10  $\mu$ M gene-specific antisense primer and 2  $\mu$ l of DNA polymerase Gene Taq which had been diluted to 1 unit/ $\mu$ l, and the resulting mixture was heated at 97°C for 5 minutes, cooled on an ice bath for 5 minutes and then a total of 28 cycles of PCR was carried out in which one cycle was comprised of the steps of 94°C for 30 seconds, 65°C for 1 minute and 72°C for 2 minutes.

After completion of the PCR, 2% agarose gel electrophoresis was carried out, the resulting gel was stained with 0.01% Cyber Green (manufactured by Takara Shuzo), and the amount of the thus stained amplified fragment was determined by Fluor Imager and used as relative expression quantity of mRNA.

In order to make a correction of the amount of mRNA, the same reaction was carried out on a house keeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene, using specific primers (SEQ ID NO:110 and NO:111) and the expression level of mRNA for each gene was corrected based on the ratio of the expression level of G3PDH mRNA, and then the average value of five cases of IgA nephropathy patients and the average value of 5 cases of healthy persons were compared and 30 gene clones having a difference in their values were

selected as genes whose expression quantity was changed in patients with IgA nephropathy. The thus selected genes are summarized in Table 1.

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TABLE 1

No	Gene	Amplification primer <sup>1)</sup>	bp <sup>2)</sup>	Expression fluctuation <sup>3)</sup>	RT-PCR primer <sup>4)</sup>	SEQ ID NO. <sup>5)</sup>	RT-PCR cycle number
1	INM063-7	FGH/OPB-2	155	12.5	43, 44	7	28
2	INP303A	FAH/OPD-5	305	9.9	45, 46	39	28
3	INM315-10	FAH/OPD-9	278	2.8	47, 48	8	35
4	INP319-3	FAH/OPD-10	135	14.4	49, 50	9	28
5	INP324A	FAH/OPD-12	197	19.9	51, 52	10	28
6	INP332A	FAH/OPD-16	137	16.6	53, 54	11	28
7	INM335-3	FAH/OPD-17	274	4.2	55, 56	12	28
8	INM336A	FAH/OPD-17	171	0.14	57, 58	13	28
9	INM351-10	FCH/OPD-4	161	1.8	59, 60	14	28
10	INP356-4	FCH/OPD-7	323	18.5	61, 62	15	35
11	INP364A	FCH/OPD-12	138	3.8	63, 64	16	28
12	INP377A	FGH/OPD-1	256	5.0	65, 66	40	28
13	INP379A	FGH/OPD-2	244	8.6	67, 68	41	35
14	INP380A	FGH/OPD-2	135	15.7	69, 70	17	35
15	INP401A	FGH/OPD-20	258	16.7	71, 72	42	24
16	INM403A	FAH/OPE-3	219	2.3	73, 74	18	28
17	INP407A	FAH/OPE-5	191	9.1	75, 76	19	28
18	INM408A	FAH/OPE-5	148	0.65	77, 78	20	28
19	INP410-5	FAH/OPE-6	306	2.0	79, 80	21	28
20	INM419-14	FAH/OPE-11	357	0.064	81, 82	22	35

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Table 1 (continued)

No	Gene	Amplification primer <sup>1)</sup>	bp <sup>2)</sup>	Expression fluctuation <sup>3)</sup>	RT-PCR primer <sup>4)</sup>	SEQ ID NO. <sup>5)</sup>	RT-PCR cycles number
21	INP429A	FCH/OPE-7	219	2.4	83, 84	23	28
22	INP431A	FCH/OPE-8	251	13.1	85, 86	24	24
23	INP438A	FCH/OPE-11	233	5.4	87, 88	25	24
24	INP444A	FCH/OPE-15	176	3.3	89, 90	26	24
25	INP451-2	FCH/OPE-4	241	14.0	91, 92	27	32
26	INP458A	FCH/OPE-11	217	9.2	93, 94	28	28
27	INP463A	FCH/OPE-19	232	18.2	95, 96	29	35
28	INP470A	FCH/OPV-4	228	5.8	97, 98	30	28
29	INP482A	FCH/OPV-10	298	9.9	99, 100	31	28
30	INP485-6	FCH/OPV-17	291	8.5	101, 102	32	28

- 1): A combination of the anchor primer with the arbitrary primer used in the differential display is shown.
- 2): The length of the amplified fragment of the differential display is shown.
- 3): Expression fluctuation is shown as the value of "the average value of mRNA expression levels in 5 cases of IgA nephropathy patients/the average value of mRNA expression levels in 5 cases of healthy persons".
- 4): The primer used in the RT-PCR is shown by the SEQ ID NO.
- 5): SEQ ID NO. of the Sequence Listing corresponding to the nucleotide sequence of amplified fragment obtained by the differential display described in Example 1 is shown.

Thus, it becomes possible to carry out diagnosis of IgA nephropathy by observing the expression levels of these genes in the leukocytes samples to be tested by RT-PCR using primers of these genes and mRNAs of the samples.

### Example 3 Cloning of whole length cDNA and analysis of each cDNA clone

#### (1) Cloning of whole length cDNA

Cloning of a cDNA containing the nucleotide sequence of amplified fragment obtained by differential display was carried out by optionally using gene trapper method, plaque hybridization of a cDNA library and 5'-RACE method. The methods are described below.

##### (A) Gene trapper method

A cDNA clone was obtained from a human leukocyte cDNA library (manufactured by Life Technologies) by the following method in which pCMV-SPORT (manufactured by Life Technologies) was used as the vector, using GENE TRAPPER cDNA Positive Selection System (manufactured by Life Technologies).

That is, clones in the cDNA library were made into single-stranded DNA (correspond to the antisense strand of cDNA) using Gene II protein and exonuclease III, and hybridization was carried out using a probe, namely a biotininated oligonucleotide specific for each gene (the sense



primer specific to each gene, used in the RT-PCR in Example 2, was used).

By allowing the biotinated probe to bind to magnetic beads to which streptoavidin had been immobilized, the above-described single-stranded cDNA hybridized with the probe was isolated.

The single-stranded cDNA clone was released from the probe, made into double-stranded DNA using a DNA polymerase and then *Escherichia coli* was transformed with the double-stranded DNA to obtain a transformant containing the cDNA clone.

Illustrative method employed was as described in the manual attached to the kit.

Each of the thus obtained transformants was suspended in 18  $\mu$ l of distilled water, the suspension was mixed with 2.5  $\mu$ l of 10  $\times$  PCR buffer, 2  $\mu$ l of 2.5 mM dNTP, 1  $\mu$ l of 10  $\mu$ M gene-specific sense primer, 1  $\mu$ l of 10  $\mu$ M gene-specific antisense primer and 0.5  $\mu$ l of DNA polymerase Gene Taq, and the resulting mixture was subjected to PCR under the same conditions as the RT-PCR, subsequently carrying out electrophoresis to isolate a transformant as the cDNA clone of interest in which a fragment having a length deduced from the positions of primers was amplified.

(B) Screening of cDNA library

Screening of cDNA clones was carried out by means of plaque hybridization using a cDNA library of leukocytes of patient with IgA nephropathy and a cDNA library of a neuroblastoma cell line NB-1.

Prior to the plaque hybridization of each library, PCR was carried out in the same manner as in Example 2, using each cDNA library as the template and using each of the gene-specific RT-PCR primers used in Example 2, and a library, in which a fragment having a length deduced from the position of the primer was amplified, was selected as the library that contains the cDNA clone of the gene of interest.

Using the library, DNAs in plaques were blotted on a nylon membrane Hybond N<sup>+</sup> (manufactured by Amersham).

Using a plasmid which contained the amplified fragment of each gene and was obtained by the differential display of Example 1, as the template, and each of the gene-specific primers used for the RT-PCR in Example 2 as a primer, PCR was carried out by adding PCR DIG labeling mix (manufactured by Boehringer Mannheim) to the reaction solution, thereby amplifying and labeling each gene-specific fragment.

Using each of the thus amplified and labeled gene-specific fragments as a probe, hybridization and detection of

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positive plaques were carried out in accordance with the manual provided by Boehringer Mannheim.

DIG Nucleic Acid Detection Kit (manufactured by Boehringer Mannheim) was used for the detection.

#### (B-1) Preparation of IgA nephropathy patient leukocyte cDNA library

A 50 ml portion of blood sample was collected from each of four patients with IgA nephropathy, and each of the blood samples was centrifuged using Polymorphprep to isolate respective leukocyte fractions. The specific method was described in the manual attached to the Polymorphprep.

Using the thus isolated leukocytes, total RNAs were prepared by employing the guanidine thiocyanate-caesium trifluoroacetate method [Methods in Enzymology, 154, 3 (1987)]. From a total of 200 ml of blood samples, 320.7 µg of total RNAs was obtained.

A 272.6 µg portion of the thus obtained total RNAs was passed through an oligo(dT) cellulose column to obtain 10.7 µg of mRNA as poly (A)<sup>+</sup> RNA.

In the same manner, 6.9 µg of mRNA was obtained from other four patients of IgA nephropathy.

Using 10.0 µg and 6.4 µg of the thus obtained respective mRNA samples, synthesis of cDNA, addition of EcoRI adapter and digestion reaction with XhoI were carried out using uniZAP-cDNA Synthesis Kit (manufactured by Stratagene),

and the resulting fragments were inserted between *EcoRI/XhoI* of  $\lambda$  Zap II by ligation to prepare a cDNA library in which the cDNA was inserted in such a direction that its 5'-end was always present in the *EcoRI* site of the vector.

The above specific method was described in the manual provided by Stratagene.

After packaging using a  $\lambda$  phage packaging kit Gigapack III Gold packaging extract (manufactured by Stratagene), *Escherichia coli* XL1-Blue MRF' was infected with the library used as the final cDNA library. The packaging and infection were carried out in accordance with the manual provided by Stratagene.

(B-2) Preparation of neuroblastoma cell line NB-1 cDNA

Using RPMI 1640 medium (manufactured by Nissui Pharmaceutical) containing 10% fetal calf serum (manufactured by Biotech International), 2% penicillin (5,000 units/ml) + streptomycin (5 mg/ml) solution (manufactured by Life Technology), 0.19%  $\text{NaHCO}_3$  (manufactured by Sigma) and 4 mM glutamine, culturing and subculturing of a neuroblastoma cell line NB-1 (*The Autonomic Nervous System*, 10, 115 (1973), available from Human Science Research Resource Bank as JCRB0621) were carried out at 37°C in an atmosphere of 5%  $\text{CO}_2$ , and  $1.25 \times 10^8$  of confluent cells were recovered.

After washing f the thus recovered cells with PBS, 10.2 µg of purified mRNA was obtained using Fast Track mRNA Isolation Kit (manufactured by Invitrogen).

A 6 µg portion of the thus obtained mRNA and 1.5 µg of NotI-primer-adapter (manufactured by Promega) were put into a container, adjusted to 7 µl by adding distilled water, heated at 70°C for 10 minutes and then rapidly cooled on an ice bath.

The thus rapidly cooled solution was mixed with 4 µl of 5 × reverse transcriptase reaction buffer (attached to the enzyme), 2 µl of 100 mM DTT, 1 µl of 10 mM dNTP and 1 µl of [ $\alpha$ -<sup>32</sup>P] dCTP (110 TBq/mmol; manufactured by Amersham) as a tracer, and the mixture was incubated at 37°C for 2 minutes, mixed with 5 µl of (1,000 units) of a reverse transcriptase, SUPERScript II RNase H<sup>-</sup> Reverse Transcriptase, and then allowed to react at 44°C for 1 hour to synthesize a cDNA.

The thus obtained reaction solution was mixed with 82 µl of distilled water, 32 µl of 5 × reaction buffer [100 mM Tris-HCl, 500 mM KCl, 25 mM MgCl<sub>2</sub>, 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM DTT, 250 mg/ml bovine serum albumin (BSA), 750 mM β-nicotinamide dinucleotide], 2.75 µl of 10 mM dNTP, 2.75 µl of [ $\alpha$ -<sup>32</sup>P] dCTP, 5.5 µl of 100 mM DTT, 2.5 µl of 6 units/µl E. coli DNA ligase (manufactured by Takara Shuzo), 11.5 µl of 3.5 units/µl E. coli DNA polymerase (manufactured by Takara Shuzo) and 2 µl of 0.6 unit/µl of E. coli ribonuclease H (manufactured by

Takara Shuzo), and the thus prepared mixture was allowed to react at 16°C for 3 hours to decompose the mRNA and obtain a double-stranded cDNA.

The reaction solution was mixed with 4.8 µl of 1 unit/µl T4 DNA polymerase (manufactured by Takara Shuzo) and subjected to 5 minutes of the reaction at 16°C to form blunt ends at both termini.

The reaction solution was mixed with 2 µl of 500 mM EDTA (pH 8.0) and 2 µl of 10% sodium dodecyl sulfate (SDS) to terminate the reaction and then extracted with phenol-chloroform to denature and remove the enzyme. An aqueous layer was obtained.

In order to remove the cDNA of 400 bp or less in length and unreacted NotI-primer-adapter and nucleotide, the thus obtained aqueous layer was put on SizeSep-400 span column (manufactured by Pharmacia) which had been equilibrated with TE buffer and centrifuged at 400 g for 2 minutes, and the resulting eluate was subjected to ethanol precipitation to recover the cDNA.

The thus recovered cDNA was dissolved by adding 5 µl (50 pmol) of EcoRI adapter (manufactured by Promega) and mixed with 40 µl of the (A) solution of Ligation Kit Ver.1 (manufactured by Takara Shuzo) and then with 5 µl of the (B) solution, and the resulting mixture was allowed at 15°C for 2

hours to effect addition of the *EcoRI* adapter to both termini of the cDNA.

The reaction solution was mixed with 40  $\mu$ l of 10 mM EDTA (pH 8.0) and heated at 65°C for 15 minutes to terminate the reaction, and then the cDNA was recovered by ethanol precipitation.

The thus recovered cDNA was dissolved in 36  $\mu$ l of distilled water and mixed with 5  $\mu$ l of 10 x reaction buffer [500 mM Tris-HCl (pH 7.6), 100 mM  $MgCl_2$ ], 2.5  $\mu$ l of 100 mM DTT, 2.5  $\mu$ l of 10 mM ATP and 4  $\mu$ l of 6 units/ $\mu$ l T4 polynucleotide kinase (manufactured by Takara Shuzo), and the mixture was allowed to react at 37°C for 30 minutes to phosphorylate the 5'-end of the added *EcoRI* adapter.

The reaction solution was mixed with 7.2  $\mu$ l of distilled water, 1.8  $\mu$ l of 5 M NaCl and 8 units (1  $\mu$ l) of *NotI*, and the mixture was subjected to 2 hours of the reaction at 37°C to cut off the *NotI* site in the *NotI*-primer-adapter.

After adding 6  $\mu$ l of 500 mM EDTA to terminate the reaction, the reaction solution was mixed with 1  $\mu$ l of 20  $\mu$ g/ $\mu$ l tRNA and then extracted with phenol-chloroform to denature and remove the enzymes. An aqueous layer was obtained. In order to remove unreacted *EcoRI* adapter, the thus obtained aqueous layer was put on SizeSep-400 span

column which had been equilibrated with TE buffer and centrifuged at 400 g for 2 minutes to recover the eluate.

The thus recovered eluate was overlaid on potassium acetate solution having a concentration gradient of from 5 to 20%, ultracentrifuged at 50,000 rpm for 3 hours and then recovered from the bottom of the centrifugation tube in 21 fractions using a peristaltic pump.

Each of the fractions was subjected to ethanol precipitation to recover cDNA, a portion of each of the thus recovered samples was subjected to agarose gel electrophoresis and then to autoradiography to measure the length of cDNA contained in each fraction, and the samples were recovered in three fractions, namely a fraction (H) containing cDNA of about 3 kb or more, a fraction (M) containing cDNA of 1 to 3 kb and a fraction (L) containing cDNA of 1 kb or less.

A 9  $\mu$ g (9  $\mu$ l) portion of a cloning vector ZAP II (manufactured by Stratagene) was mixed with 10  $\mu$ l of 10 x H restriction enzyme buffer (manufactured by Takara Shuzo), 75  $\mu$ l of distilled water and 90 units (6  $\mu$ l) of EcoRI, and the mixture was subjected to 2 hours of the reaction at 37°C.

The reaction solution was mixed with 1  $\mu$ l of 5 M NaCl and 40 units (5  $\mu$ l) of NotI, allowed to react at 37°C for 2 hours, and further mixed with 8 units (1  $\mu$ l) of NotI and



again subjected to 1 hour of the reaction at 37°C to cleave the *EcoRI* site and *NotI* site of the vector.

The reaction solution was mixed with 100  $\mu$ l of 2 M Tris-HCl (pH 8.0) and 1 unit (2  $\mu$ l) of *E. coli* C75 alkaline phosphatase (manufactured by Takara Shuzo) and allowed to react at 60°C for 30 minutes to dephosphorylate the 5'-ends cleaved by *Eco*RI and *Not*I the vector, and then these enzymes were removed by repeating phenol-chloroform extraction twice.

After removal of the enzymes, chloroform extraction was carried out and the resulting water layer was subjected to ethanol precipitation to recover the vector DNA which was subsequently dissolved in TE buffer.

Each of the cDNA samples recovered in three fractions was mixed with 1  $\mu$ g of the vector DNA and subjected to ethanol precipitation, and the thus recovered vector DNA and cDNA were dissolved in 4  $\mu$ l of a ligase buffer [100 mM Tris-HCl (pH 7.6), 5 mM  $MgCl_2$ , 300 mM NaCl], mixed with 4  $\mu$ l of the (B) solution of Ligation Kit Ver.1 and then allowed to react at 26°C for 10 minutes to bind the cDNA to the vector DNA.

A 4  $\mu$ l portion of each of the reaction solutions was subjected to packaging using a  $\lambda$  phage packaging kit, Giga-Pack Gold II (manufactured by Stratagene). The reagents and methods were described in the manual attached to the kit.

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*E. coli* XL1-Blue MRF' was infected with the thus obtained phage and the titer was measured. Thereafter, the cDNA library was amplified once by growing the phage on a plate medium and recovering it in SM buffer and used as the final cDNA library. The measurement of titer and amplification of library were carried out in accordance with the manual attached to the  $\lambda$  phage packaging kit. A library prepared from the (E) fraction containing cDNA of about 3 kb or more was used for the screening of the present invention.

(C) 5'-RACE

5'-RACE of the IgA nephropathy patient cDNA prepared in the above method (B) was carried out using 5'-RACE System ver.2 (manufactured by Life Technologies). The specific method was described in the manual attached to the kit.

Using the above methods (A) to (C), cDNA cloning of the five genes shown in Table 2 was achieved.



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Nucleotide sequence of the cDNA moiety of each of the thus obtained cDNA clones was determined using 377 DNA Sequencer manufactured by Perkin Elmer. Determination of the nucleotide sequence was carried out using Dye cycle sequencing FS Ready Reaction Kit in accordance with the manual attached to the kit. Additionally, the nucleotide sequence was translated into amino acid sequence by three frames to examine whether an open reading frame (ORF) composed of 100 or more amino acids is present.

(1) INP303A

A cDNA clone GTINP303A-41a was obtained by the gene trapper method, but this was considered to be an incomplete cDNA clone because of the absence of ORF, which corresponds to 100 or more amino acids, in the nucleotide sequence of the cDNA.

In order to obtain a full-length length cDNA clone, 5'-RACE was carried out using specific primers (nucleotide sequences are shown in SEQ ID NO:108 and NO:109) which correspond to a moiety close to the 5'-end of GTINP303A-41a to obtain cDNA clone INP303A-R1. Also, since a part of the cDNA nucleotide sequence of GTINP303A-41a was not able to determine, another cDNA clone INP303A-ph1-3 was obtained from an NB-1 cDNA library by plaque hybridization.

By combining nucleotide sequences of these cDNA clones thus obtained, a 4,276 bp nucleotide sequence of the cDNA of INP303A was determined as shown in SEQ ID NO:1.

The nucleotide sequence of a fragment obtained by differential display (SEQ ID NO:39) coincided with the complementary chain nucleotide sequence corresponding to the positions 2,797 to 3,101 of SEQ ID NO:1. Therefore, it was considered that the anchor primer was not annealed to the 3'-end poly(A) sequence of mRNA but to the complementary chain of a sequence having a series of T and existing in the positions 2,782 to 2,795 of SEQ ID NO:1.

An ORF corresponding to 239 amino acids (corresponds to the positions 53 to 742 of SEQ ID NO:1, the amino acid sequence is shown in SEQ ID NO:33) was found in the nucleotide sequence of the cDNA of INP303A-R1.

When the amino acid sequence of the ORF was compared with an amino acid data base, it was found that this sequence has a homology with C40H1 which was estimated to be a protein encoded by a Nematoda genomic gene clone C40H1, mouse cytoplasmic polyadenylation element binding protein (CPEBP) and Drosophila orb gene.

It was found also that an amino acid sequence just downstream of the region where these proteins showed a homology with the INP303A protein also showed a homology with the amino acid sequence encoded by the nucleotide sequence of

positions 3,346 to 3,577 of SEQ ID NO:1. Therefore, it was assumed that this cDNA is a result of abnormal splicing in which a 2,689 bp nucleotide sequence (corresponds to positions 713 to 3,352 in SEQ ID NO:1) which seems to be an intron originally remained in the nucleotide sequence of INP303A.

It was found that the nucleotide sequence of a fragment which was obtained by the differential display and whose expression quantity increased in IgA nephropathy patients is present in this insertion sequence and the amount of mRNA which caused such an abnormal splicing increases in IgA nephropathy patients. It is highly possible that a protein translated from an mRNA which caused the abnormal splicing does not exert its original function, because its amino acid sequence at and after the 220 position is different from the original protein encoded by INP303A, namely a protein (295 amino acids) encoded by a nucleotide sequence resulting from the elimination of intron deduced from the a homology.

## (2) INP377A

Nucleotide sequence of the cDNA of cDNA clone GTINP377A-46C was determined by the gene trapper method, with the thus obtained nucleotide sequence shown in SEQ ID NO:2.

When the nucleotide sequence of INP377A cDNA was compared with a nucleotide sequence data base, it was found

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that a sequence of the positions 1 to 552 of a human gene LUCA15 (GenBank accession No. U23946) which has a homology with a Drosophila cancer inhibition gene 8x1 coincides with the 50 to 527 position nucleotide sequence and 1,010 to 1,083 position nucleotide sequence of GTINP377A-46C. Consequently, it was assumed that GTINP377A-46C is a cDNA clone in which an intron of LUCA15 remained by an abnormal splicing.

A nucleotide sequence (SEQ ID NO:40) of a fragment obtained by the differential display method coincided with the nucleotide sequence of a complementary chain corresponding to the positions 759 to 1,014 of SEQ ID NO:2. Accordingly,, it was considered that the anchor primer was not annealed to the 3'-end poly(A) sequence of mRNA but to the complementary chain of a sequence having a series of T and existing in the positions 745 to 757 of SEQ ID NO:2. Since the nucleotide sequence of the fragment is considered to be present in the nucleotide sequence which seems to be an intron of LUCA15, it is probable that the amount of mRNA which caused such an abnormal splicing increases in IgA nephropathy patients.

It is highly possible that the protein of 143 amino acids (the amino acid sequence is shown in SEQ ID NO:34) which is encoded by GTINP377A-46C does not exert its original function, because its amino acid sequence at and after the

137 position is different from the original protein (815 amino acids) encoded by LUCA15 cDNA.

(3) INP379A

A cDNA clone of INP379A, namely PHINP379A-16-2, was obtained by plaque hybridization of a cDNA library prepared from leukocytes of IgA nephropathy patients.

When the nucleotide sequence of the cDNA was determined, the *Xho*I site and poly T sequence were present in a side which was thought to be the 5'-end, so that it was considered that this is a clone in which cDNA was inserted into the vector in the opposite direction.

Consequently, a nucleotide sequence complementary to the thus obtained nucleotide sequence, which is the original nucleotide sequence of the cDNA, is shown in SEQ ID NO:3.

The nucleotide sequence of a fragment obtained by differential display (SEQ ID NO:41) coincided with the nucleotide sequence of the positions 2,706 to 2,949 of SEQ ID NO:3. An ORF corresponding to 104 amino acids (the amino acid sequence is shown in SEQ ID NO:35) was present in this nucleotide sequence.

Since no sequences having a homology with this amino acid sequence were found in the amino acid sequence data base, this cDNA was considered to be a gene which encodes a novel protein.



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(4) INP401A

Two cDNA clones of INP401A, namely PHINP401A-8-1 and PHINP401A-14-1, were obtained by plaque hybridization of a cDNA library prepared from leukocytes of IgA nephropathy patients.

When nucleotide sequences of both cDNAs were determined, it was found that both sequences contained the same ORF corresponding to 133 amino acids, except for only one different base and therefore only one corresponding amino acid. Also, since both sequences were different from each other with regard to their nucleotide sequences of 5'-side non-translation region and 3'-side non-translation region, the presence of mRNAs having different polymorphism and splicing of the gene was assumed.

The nucleotide sequence of PHINP401A-8-1 is shown in SEQ ID NO:4, the nucleotide sequence of PHINP401A-14-1 in SEQ ID NO:5, the amino acid sequence of the protein encoded by PHINP401A-8-1 is shown in SEQ ID NO:36, and the amino acid sequence of the protein encoded by PHINP401A-14-1 in SEQ ID NO:37.

The nucleotide sequence of a fragment obtained by differential display (SEQ ID NO:42) coincided with the complementary chain nucleotide sequence corresponding to the positions 960 to 1,217 of SEQ ID NO:4 and the complementary chain nucleotide sequence corresponding to the positions

1,313 to 1,570 of SEQ ID NO:5. Accordingly,, it was considered that the anchor primer was not annealed to the 3'-end poly(A) sequence of mRNA but to the complementary chain of a sequence having a series of T and existing in the positions 947 to 959 of SEQ ID NO:4 or the positions 1,302 to 1312 of SEQ ID NO:5.

The nucleotide sequence of a fragment which was obtained by the differential display and whose expression quantity increased in IgA nephropathy patients was found to have a nucleotide sequence complementary to the nucleotide sequences of PHINP401A-8-1 and PHINP401A-14-1.

The homology of the proteins encoded by PHINP401A-8-1 and PHINP401A-14-1 was examined, but no sequences having a homology were found in the amino acid sequence data base. Accordingly, they were considered to encode novel proteins.

An analysis of hydrophilic property deduced from the amino acid sequence showed a possibility that the protein encoded by INP401A is a secretory protein, and, in that case, the 1 to 15 position amino acid sequence of SEQ ID NO:36 or NO:37 was assumed to be the signal peptide.

(5) GTINP322A-21

An attempt was made to obtain a cDNA clone of INP332A by the gene trapper method; however, nucleotide sequence of the thus obtained cDNA clone GTINP322A-21 contained no nucleotide sequence which coincides with the amplified



As clear from the above examples, the diagnosis, treatment or prevention of IgA nephropathy can be effected using the DNA, protein and antibody of the present invention.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

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